-- 36. The method according to claim 25, wherein the fibronectin polypeptide comprises an alternatively spliced non-type III connecting segment of fibronectin. --

REMARKS

The Office Action

Claims 25, 28 and 31-35 have been rejected under 35 U.S.C. §112, first paragraph. Claims 31 and 32 have been rejected under 35 U.S.C. §112, second paragraph.

The Specification and Pending Claims

The specification has been amended to reflect the present status of the parent applications.

New claim 36 has been added. Support for this new claim can be found, e.g., at page 8, lines 15-17 of the specification.

No new subject matter has been added. Upon entry of this amendment, claims 25, 28, and 31-36 will be pending.

In view of the claim amendments made herein and the following remarks, Applicant respectfully submits that the present application is in condition for allowance.

Clarification of Priority Date

In paragraph 3 of the outstanding Office Action, the Examiner notes that a priority date of May 22, 1995 has been set for claims drawn to EILDV in the instant application.

Applicant respectfully disagrees with the Examiner's determination of the priority date for the EILDV claims, since support for these claims can be found in the priority application, USSN 08/029,330, filed on February 9, 1993. The Examiner is directed to page 4, lines 36-38 through page 5, line 1 of USSN 08/029,330, which provides:

> Also contemplated are soluble forms of the natural binding proteins for VLA 4, including soluble VCAM-l or VCAM-1 peptides as well as fibronectin, fibronectin having an alternatively spliced non-type III connecting segment and fibronectin peptides containing the amino acid sequence EILDV or a similar conservatively substituted amino acid sequence. (see also, page 9, lines 27-33 of USSN 08/029,330)

In view of the above, the Examiner is respectfully requested to reconsider the priority date for the EILDV claims and to assign a date of February 9, 1993 based on the disclosure of USSN 08/029,330.

Correction of the Drawings

In paragraph 5 of the outstanding Office Action, the drawings are objected to as informal because of the recitation of "PSS ->", since, according to the Examiner, this term is not defined in the Figures or in the Brief Description of the Drawings.

Applicant will defer correcting the drawings until the pending claims are indicated otherwise allowable.

Rejection of Claims 25, 28 and 31-35 Under 35 U.S.C. 112, First Paragraph

In paragraphs 7-9 of the outstanding Office Action, the Examiner has rejected claims 25, 28 and 31-35 under 35 U.S.C. 112, first paragraph, "as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention." According to the Examiner:

> One cannot extrapolate the teaching of the specification to the enablement of the claims because the art of diabetes treatment is clearly not predictable or developed and because the specification does not provide teachings to establish effective dosages or methods of administration of the claimed fibronectin polypeptides [e.g., fibronectin peptides containing the amino acid sequence EILDV] for treatment of insulin dependent (type I) diabetes and does not teach how to insure that the polypeptides interact at the proper site of action and do so at a sufficient concentration and for a sufficient period of time....The specification clearly fails to provide any details about methods of administering the claimed fibronectin polypeptide [e.g., fibronectin peptides containing the amino acid sequence EILDV], targeting the polypeptide to the appropriate cells, stability of a polypeptide *in vivo*, or appropriate doses of polypeptide of the invention to achieve the desired effect, nor does the specification provide working examples which would provide guidance to one skilled in the art and no evidence has been provided which would allow one of skill in the art to use the claimed method with a reasonable expectation of success.

Applicant respectfully traverses the above-quoted rejection. The claims, as presently pending, are directed to a method for treating diabetes, in a prediabetic mammal, by

administering a soluble fibronectin polypeptide, e.g., a fibronectin polypeptide comprising an EILDV motif, a chimeric fibronectin-toxin molecule, or a fibronectin polypeptide comprising an alternatively spliced non-type III connecting segment of fibronectin.

The instant specification provides sufficient guidance to enable the claimed methods. The present invention is based, in part, on the discovery that blocking the interaction between the Very Late Antigen-4 receptor VLA-4 ($\alpha 4\beta 1$) and its alternate ligands, Vascular Cell Adhesion Molecule VCAM-1 or fibronectin, is sufficient to significantly reduce the incidence of diabetes in a rodent model of diabetes. Diabetes, as is the case for a number of immune conditions, such as transplant rejection and autoimmune conditions, involves the migration and activation of immune cells, e.g., lymphocytes, macrophages and dendritic cells, to inflammatory sites. In the case of diabetes, the immune cells typically destroy islet β cells in the pancreas. Cell trafficking to inflammatory sites is regulated, *inter alia*, by an intricate inteplay of integrins and their counter-ligands. For example, the combination of LFA-1 and VLA-4, and Mac-1 and VLA-4 on the surface of lymphocytes and macrophages, respectively, and by their counter-ligands ICAM (for LFA-1 and MAC-1) and VCAM and fibronectin (for VLA-4).

The anti-VLA-4 antibodies and VCAM-IgG fusions used in the working examples of the instant application demonstrate that VLA-4 inhibitors can be used to ameliorate diabetes *in vivo*. Specific effective dose ranges and modes of administration of these VLA-4 inhibitors are provided in detail in the instant application (see e.g., page 13, lines 4-26). Working examples describing the effectiveness of these inhibitor at the dosages disclosed are extensively described in the application, e.g., Examples 1-5). The Examiner has not given any specific rationale or basis for assuming that this guidance is insufficient or ineffective.

Applicant submits that other agents described in the instant specification, such as fibronectin polypeptides, could also be effectively used to treat diabetes by simply following the teachings of the specification. The determination of dosage is a routine matter for one of ordinary skill in the art. The present application provides that dose ranges of non-antibody (e.g., peptide) inhibitors can be between molar equivalent amounts of the antibody dosages discloses (see page 13, lines 12-13 of the specification). Furthermore, the instant application provides an example of how one would optimize or determine dosages by monitoring the coating of VLA-4 positive cells by the inhibitors over time after administration at a given dose *in vivo* (see e.g., page 13, lines 15-26). For example, the specification provides that preferred dosages are such that produce a detectable coating of the vast majority of VLA-4 positive cells. Peripheral blood

mononuclear cells contained in the sample of the individual's peripheral blood can be probed for the presence of the inhibitor *in vitro* (or *ex vivo*) using a second reagent to detect the administered inhibitor, e.g., using a fluorochrome labeled anti-inhibitor antibody which is the measured using fluorescent activated cell sorter (FACS) analysis. Alternatively, the presence of the administered inhibitor may be detected *in vitro* (or *ex vivo*) by the inability or decreased ability of the individual's cells to bind the same inhibitor which has been itself labeled (e.g., by fluorochrome). Following this guidance provided in the present application, one of ordinary skill could determine the adequate fibronectin polypeptide dosage as a routine matter.

In another portion of this rejection, the Examiner supports the rejection for lack of enablement of claim 32, which is directed to a fibronectin polypeptide fused to a toxin moiety, by stating that:

> It would appear that the result of the treatment as claimed, that is with a toxin/fibronectin molecule, would result in the destruction of all lymphocytes and macrophages expressing VLA-4 which would result in a general immunosuppression of the prediabetic mammal or the mammal with partial beta cell destruction. It cannot be predicted how a general immunosuppression would treat insulin dependent (type I) diabetes, especially in a prediabetic mammal.

Applicant respectfully traverses the above-quoted portion of this rejection.

The VLA-4 receptor has been shown to have at least two distinct affinity states, a high and a lower affinity state, depending on the level of activation (see e.g., Jakubowski, A. et al. (1995) Cell Adhesion and Communication 3:131-142). When present on activated cells, e.g., pathogenic immune cells, VLA-4 shows higher affinity for its ligands, e.g., VCAM and fibronecin, compared to the affinity displayed by VLA-4 on resting cells. Therefore, by adjusting the effective concentration of the fibronectin/toxin fusion, it is possible to selectively target those pathogenic cells, leaving the resting cells substantially unaffected.

In yet another aspect of this rejection, the Examiner further rejects claims 31 and 32 under 35 U.S.C. §112, first paragraph, because the specification:

> while being enabling for a polypeptide comprising a fibronectin polypeptide and a toxin moiety, does not reasonable provide enablement for a fibronectin polypeptide that is a component of a chimeric molecule or said chimeric molecule wherein the chimeric molecule further comprises a toxin moiety....
>
> One cannot extrapolate the teaching of the specification to the scope of the claims because the specification provides no guidance on the production of a chimeric molecule other than a VCAM Ig fusion protein and provides no guidance on what the "optionally second peptide" might be.
>
> It is clear that modification of the fibronectin polypeptide in the production of a "chimeric molecule" would require addition of amino acids and it could not be predicted what affects these additional residues would have on the specificity and function of the fibronectin polypeptide. Certainly it would be expected that three dimensional conformation changes in the polypeptide would occur and it could not be predicted from the specification whether all types of "chimeric" molecules would retain the ability to function as claimed.

This portion of the rejection is respectfully traversed. The Examiner has provided no specific arguments as to why one would expect that addition of another moiety would destroy the activity of the chimeric molecule. It is known in the art that moieties can be combined together to form, e.g., fusion proteins, multi-specific proteins (bi- tri-specific antibodies). The determination of which linker to use to enable the proper conformation of the attached moieties in the chimeric molecule is a routine matter for one of ordinary skill in the art.

In view of the foregoing, reconsideration and withdrawal of the rejection of the pending claims is respectfully requested.

Rejection of Claims 31 and 32 Under 35 U.S.C. §112, Second Paragraph

Claims 31 and 32 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. According to the Examiner:

> Claims 31 and 32 are indefinite in the recitation of "chimeric molecule" because the exact meaning of the word chimeric is not known. The term chimeric is generic to a class of molecules which are products of genetic shuffling of several other active proteins. The term encompasses soluble fibronectin polypeptides fused to other proteins as well as soluble fibronectin polypeptides wherein any domain of the polypeptide is substituted by corresponding regions from other VLA-4 blocking agents.

Applicant respectfully traverses this rejection. As pointed out by the Examiner, the term chimeric molecule is art-recognized to mean a class of molecules, which comprise different kinds of molecules linked to each other, e.g., fusions or replacements. The fact that both fusions and replacements are encompassed by this term does not render the term indefinite. Accordingly, reconsideration and withdrawal of this rejection is respectfully requested.

SUMMARY

The above rejections and objections are either improper or do not pertain to the claims as newly amended and should be withdrawn. The present claims are in condition for allowance.

The amendments and cancellations made herein are not an acquiescence to the Examiner's rejections. Applicant reserves the right to prosecute similar claims in a related application.

If a telephone conversation with Applicant's Attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call Applicant's Attorney at (617) 542-5070.

The Commissioner is hereby authorized to charge payment of any additional fees or credit any overpayment to Deposit Account No. 06-1050.

Respectfully submitted,
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Appendix A

25. A method for the treatment of insulin dependent (type I) diabetes comprising administering to a prediabetic mammal, or a mammal having partial β cell destruction, a composition comprising a soluble fibronectin polypeptide, in an amount effective to treat diabetes.

28. The method according to claim 25, wherein the fibronectin polypeptide comprises an EILDV motif.

31. The method according to claim 25, wherein the fibronectin polypeptide is a component of a chimeric molecule.

32. The method according to claim 31, wherein the chimeric molecule further comprises a toxin moiety.

33. The method according to claim 25, wherein the mammal is prediabetic.

34. The method according to claim 33, wherein the prediabetic mammal is a human.

35. The method according to claim 25, wherein the mammal has partial β cell destruction.

36. The method according to claim 25, wherein the fibronectin polypeptide comprises an alternatively spliced non-type III connecting segment of fibronectin.

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Appendix B

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Review

Lessons from the NOD mouse for the pathogenesis and immunotherapy of human Type 1 (insulin-dependent) diabetes mellitus

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Summary. Suitable animal models of human Type 1 (insulin-dependent) diabetes mellitus have long been sought, in particular a model that would permit detailed histological and immunological investigation of changes in the islet preceding the metabolic disorder. This would allow hypotheses as to pathogenesis of the condition to be examined and interventions such as immunotherapy to be tested. The most widely studied models include the low-dose streptozotocin induced diabetic mouse and the BB rat, but both differ in important respects from the human disease. In this review we describe one highly successful model, the non obese diabetic mouse. Selected aspects of pathogenesis and immunotherapy are presented and analogies with human Type 1 diabetes discussed.

Key words: Non obese diabetic (NOD) mouse, pathogenesis Type 1 (insulin-dependent) diabetes mellitus, immunotherapy Type 1 diabetes.

The NOD mouse was derived from a cataract-developing substrain of the outbred JcI-ICR mouse by selective breeding from 1974 to 1980 [1]. Diabetes develops spontaneously between the 12th and 30th week of age, with an onset characterized by polydipsia, glycosuria, rapid weight loss, hyperglycaemia and ketoacidosis (Table 1). The onset of hyperglycaemia is preceded by insulitis, progressive B-cell destruction and decreasing circulating insulin levels leading to insulin dependency [2–4]. Without insulin treatment the animals die within 4 to 8 weeks (unpublished observations). Thus, clinical and pathological features in the NOD mouse closely resemble human Type 1 (insulin-dependent) diabetes mellitus. Since all conclusions drawn from animal models are, however, based on analogy with human disease, the analogy needs detailed validation. For this reason we describe similarities and differences relating to pathogenesis and immunotherapy in the NOD mouse and human Type 1 diabetes.

Genetic background

Continued in-breeding of the strain has resulted in high genetic uniformity as shown by morphology, allele distribution of enzymes and other proteins, and immunological studies including mixed lymphocyte reaction and skin grafting [5]. Based on this, the genetic background of insulitis and overt diabetes has been investigated by backcross experiments with C57BL, NZB mice and a non obese non diabetic subline (NON) of the same origin as the NOD [6–8]. The results indicate three recessive diabetogenic genes, two of which are non MHC-linked. One controls the development of severe insulitis and appears to be incompletely dominant, and the other is involved in the progression to diabetes, probably mediated by a lack of specific suppressor cells. The third, MHC linked, gene is not required for insulitis but apparently influences the autoimmune response [7]. It has been suggested that the NOD mouse has a unique class II MHC, which may lead to the autoimmune insulitis [9]. Furthermore, treat-

Table 1. Comparison of clinical features at onset of diabetes in the human and the NOD mouse

	Type 1 (insulin-dependent) diabetes mellitus	
	human	NOD mouse
Weight loss	Present	Present
Polydipsia	Present	Present
Polyuria	Present	Present
Hyperglycaemia	> 15 mmol/l	20–30 mmol/l
Ketoacidosis	Common	Less severe
Serum insulin	Very low	Very low
Outcome without insulin	Lethal	Lethal
Sex preponderance	Female ≧ male	Female ≫ male

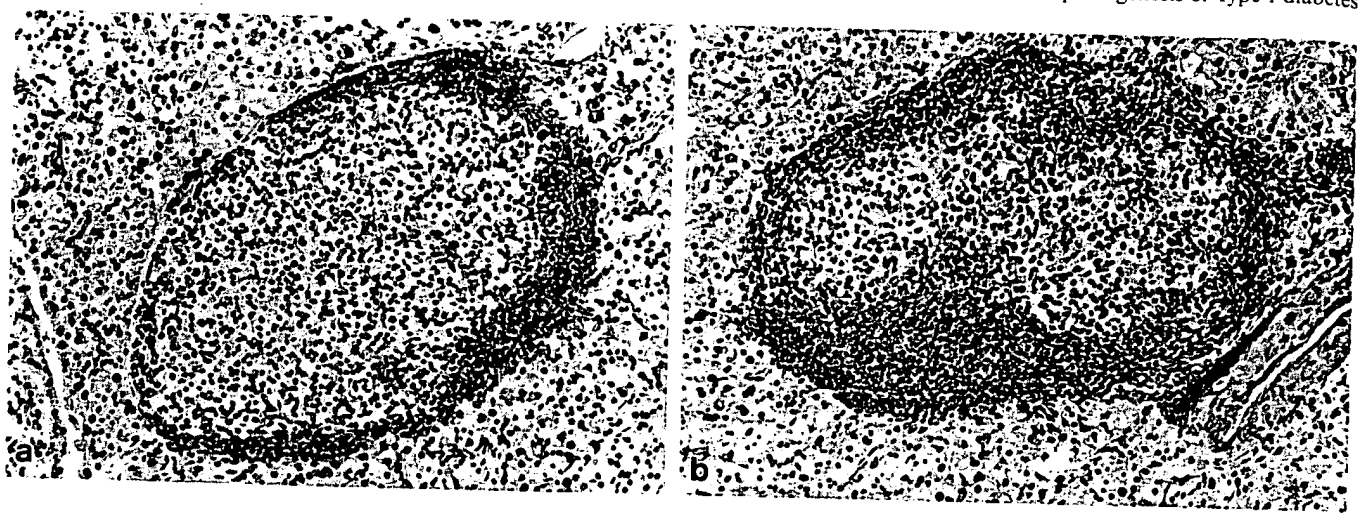

Fig. 1a and b. Micrographs of islets of Langerhans in a 20-week-old female NOD-mouse showing periinsulitis (**a**), followed by invasion of the islet by lymphocytes penetrating the capsule (**b**) (Magnification 145 ×, haematoxylin and eosin staining)

ment with anti-I-A monoclonal antibody prevented diabetes in NOD mice [10].

Human Type 1 diabetes is associated with MHC products [11], most closely linked with the HLA DQ region [12]. In mice the equivalent to DQ-beta is the A-β chain, and this has interesting similarities with human diabetes [13]. Back-cross experiments have shown that homozygosity at this gene is necessary for the development of diabetes. The NOD A-β allele is unique in the species in having serine in position 57 instead of aspartic acid (Asp). Similarly in humans DQ-β Asp 57 negative homozygosity is found in 90% of Caucasian Type 1 diabetic patients, whereas, Asp 57 positive homozygosity at DQ-β gives almost complete protection from Type 1 diabetes [13].

Despite strong evidence for an association with a genetic factor or factors, the concordance rate for Type 1 diabetes is surprisingly low in identical twins (30–50%), suggesting that susceptibility is inherited rather than the expressed disease [14]. The NOD mouse resembles man in this respect, since the animals are genetically identical but not all develop diabetes. Abnormal immunological parameters including islet cell antibodies (ICA) and increased numbers of circulating activated T cells are, however, concordant in human twin studies [15, 16] and NOD mice also have a concordant immunological process, as shown by the fact that all females and more than 90% of males exhibit insulitis [7, 17, 18]. The incidence of diabetes is, however, at least twice as high in female NOD mice than in males, and castration experiments suggest that this difference is related to female sex hormones [17]. Castration of mice up to the age of 7 weeks results in an increase of diabetes incidence in males and a decrease of incidence in females.

Administration of testosterone prevents diabetes in castrated animals, whereas oestradiol raises the incidence of diabetes in castrated animals of both sexes. The rate of development of diabetes is also influenced by diet [17]. Although hormonal and dietary influences have not been shown in human diabetes, human Type 1 diabetes and the NOD model suggest that genetic factors predispose to the autoimmune disorder, but have limited importance for clinical expression of the disease.

The genetic uniformity in this inbred strain has great advantages in the experimental situation as a guarantee of identity, but for the same reason has limited relevance to the human situation. Thus, even though 95% of Type 1 diabetic patients are HLA DR3 or DR4 positive [20], there is marked genetic heterogeneity. The mechanism of inheritance of the disease in the NOD mouse cannot, therefore, be identical to that in man, although it constitutes one of the possible alternatives.

Histopathology

Insulitis is the pathological hallmark recent onset Type 1 diabetes and is observed in the NOD mouse from at least the 4th week of age [21, 22]. The earliest change is periinsulitis adjacent to the pancreatic ducts, followed by invasion of the islet capsule by small lymphocytes which penetrate the islet (Fig. 1). The final stage is characterized by small islets from which B-cells have disappeared, with resolution of insulitis. The different stages of this process can, however, be found within the same pancreas at any age. Phenotyping of lymphocyte subsets involved in the insulitis has produced conflicting results [23–26]. We have found that monocytes and B-lymphocytes are the predominant cell population [25]. Previous studies have reported L3T4 cells (mainly helper/inducer) and MHC class-II cells as the most represented subsets [23, 26]. Within the T-lymphocyte population L3T4 cells are more frequently found than Lyt-2 cells (mainly cytotoxic/suppressor) [24].

Table 2. Comparison of morphological features in human diabetes and the NOD mouse

	Type 1 (insulin-dependent) diabetes mellitus	
	Human	NOD mouse
Periinsulitis/insulitis	Present	Present
Insulitis in subjects without diabetes	?	Present
Small islets lacking B-cells at the end stages	Present	Present
Lymphocytic infiltration in other organs	Rare	Present

The prevalence of insulitis is high in humans who have died soon after the onset of Type 1 diabetes, and in one early study insulitis was present in 16 of 23 who died within 6 months of onset [27, 28] and in 47 out of 60 patients with a diabetes duration of less than one year [29]. The smouldering nature of the process is equally apparent, with normal islets, insulitis and "end-stage" islets depleted of B cells within the same histological field. While there is still some controversy concerning the prevalence of insulitis in man, there is agreement on the histological pattern of lymphocytic infiltration (Table 2). As in the NOD mouse, insulitis develops in man as periinsulitis and progresses to infiltration of the islets and B-cell destruction [28]. There has been only one report concerning the phenotype of lymphocyte subsets, based on the pancreas of a child who died at the time of diagnosis [30]. The majority of infiltrating lymphocytes were T-cells, predominantly CD8 positive although other inflammatory cells were present. Thus, despite possible differences between the lymphocyte subsets infiltrating the islets, the NOD mouse is a good model from the histopathological point of view. Diabetes has a strong female preponderance in the NOD mouse (70% vs 20% at 30 weeks of age) but insulitis is present to a similar degree in both sexes. Thus, about 80% of males and 30% of females show insulitis without developing diabetes up to the 30th week of age [4]. In the human situation we remain ignorant as to the time of development of insulitis prior to the disease, although it is assumed to coincide with the appearance of ICA and other autoimmune markers, and it is not known whether individuals with insulitis inevitably progress to diabetes.

In NOD mice lymphocytic infiltration is not restricted to the islets but occurs also in salivary tissue [31] and occasionally in the thyroid and adrenal glands [32], suggesting a wider disturbance of immune tolerance in this animal. Type 1 diabetes is also associated with overt polyendocrine disease and there is an increased prevalence of autoantibodies to thyroid, adrenal or gastric parietal cells, although figures concerning this vary [33]. Infiltration of salivary glands has not, however, been described in human diabetes.

Immunological observations

Autoantibodies

ICA have been found in about 50% of NOD mice up to the 21st week of age but tend to disappear later [34, 35]. Islet cell surface antibodies (ICSA) appear at 3-6 weeks, reach peak incidence and titre at around 12-18 weeks, and decline thereafter [26, 34]. There is no evidence that these autoantibodies are directly involved in B-cell destruction, and both ICA and ICSA might be secondary to islet cell destruction and massive release of cellular antigen, a view which accords with the time course of insulitis in this animal model. Insulin autoantibodies (IAA) have also been reported; they may antedate insulitis [35] and are present in almost all animals later in life [34]. Finally, autoantibodies which immunoprecipitate a 64.000 mol.wt.islet antigen have recently been described [36]. As in humans, the pathogenetic relevance of these autoantibodies remains uncertain, and the prognostic significance of ICA, ICSA, insulin and 64 kilodalton autoantibodies has yet to be investigated in the NOD mouse.

Cell mediated immunity

Several successful attempts have been made to transfer insulitis and diabetes via lymphocytes derived from NOD mice, using a variety of protocols. Diabetes appears within a few weeks of lymphocyte transfusion, providing further support for the autoimmune hypothesis. Recipient animals were either newborn or very young normal NOD mice [37], totally irradiated NOD mice [38, 39], or athymic nude mice of NOD origin [40]. Despite these differences, similar results were obtained with regard to the age of lymphocyte donors, and 100% successful transfer of diabetes/insulitis can only be achieved with lymphocytes from mice at least 16-19-weeks-old [37, 39]. Interestingly the transfer can be made with either diabetic or non-diabetic donor lym-

Table 3. Comparison of immunological features of Type 1 (insulin-dependent) diabetes in humans and the NOD mouse

	Type 1 diabetes mellitus	
	Human	NOD mouse
Insulin autoantibodies	Present	Present
Islet cell antibodies	Present	Present
Islet cell surface antibodies	Present	Present
Islet cell specific cellular immunity	Present	Present
Abnormal T-helper/T-suppressor	Present	Present
Major T-lymphocyte subset in the insulitis	$CD8^+$	$L3T4^+$
Aberrant expression of class II MHC on insulin positive cells	Present	?

phocytes. In older non-diabetic recipients (i.e. >25 weeks) the transfer is much less effective and only 5 of 16 developed diabetes [39]. When separated lymphocyte subsets were used, both Lyt-2^+ (mainly cytotoxic/suppressor) and L3T4^+ (mainly helper/inducer) cells appeared to be necessary. In addition, both subsets should be derived from a donor of appropriate age (16 to 19 weeks) as shown in transfer experiments in which L3T4^+cells from an appropriate donor were reconstituted with Lyt 2^+cells from a 6-week-old donor (or vice versa) but failed to induce diabetes when transferred. It was further shown that newborn mice are susceptible to transfer until the 3rd week (females) and the 5th week (males).

The NOD mouse, therefore, appears susceptible to the transfer of diabetes until the time at which insulitis develops spontaneously. At approximately 16 to 19 weeks the animals acquire the ability to transfer the disease with lymphocytes, but this capacity is often lost in non-diabetic mice from the 25th week onwards. These findings may reflect time dependent differences in the development of necessary lymphocyte subsets (i.e. T-helper/inducer first, antigen-specific effector second and T-suppressor cells, last). If this is the case B-cells might disappear too rapidly for the induction of T-suppressor cells in animals which develop diabetes, whereas animals with slower destruction of B cells may produce sufficient specific T-suppressor cell activity to protect themselves from further B-cell loss.

A variety of cellular cytotoxicity systems have been investigated in search of an active effector cell mechanism in the NOD mouse. Direct cellular cytotoxicity (CTL) was increased as compared to ICR mice using Balb/c islets as targets in a chromium release assay. Antibody dependent cellular cytotoxicity (ADCC) and natural killer (NK) cell activity have been tested in NOD and ICR mice. Both ADCC against chicken erythrocytes in the presence of anti-chicken erythrocyte antibodies and NK activity against Chang liver cells are decreased in the NOD mouse [41]. Another interesting observation is that athymic nude mice with NOD background [40] or NOD mice undergoing neonatal thymectomy [18] did not develop insulitis and diabetes - suggesting a pivotal role for T-lymphocytes in the autoimmune process. Administration of monoclonal antibodies (mAb) specific to some lymphocyte surface markers can block function or destroy the corresponding cell subset. Thus, treatment with anti Thy 1.2 mAb (T cells) prevents diabetes but does not influence the progression of insulitis [40]. Administration of L3T4 mAb abolishes insulitis and diabetes [42, 43]. In addition, Lyt 2^+cells (suppressor/cytotoxic) and macrophages are necessary for the development of insulitis since treatment with anti-Lyt2 antibody and silica particles prevents B-cell destruction [44].

Thus, macrophages and Lyt2^+cells are required for induction of the autoimmune process by appropriate antigen presentation and for generation of specific effector cells, respectively. On the other hand, cyclophosphamide (known to impair T-suppressor cells) promotes overt diabetes and increases its incidence in the NOD mouse [45]. These data suggest the presence of specific T-suppressor cells in the NOD mouse, although these are clearly not efficient enough to maintain tolerance in all cases.

Aberrant expression of HLA class II on B cells has been claimed to play an important role in the initiation of the autoimmune process leading to diabetes [46]. Class II expression was found in a child who died soon after clinical presentation [30] and confirmed by an immunohistological study of formalin fixed paraffin embedded tissue from post mortem cases with recent onset of diabetes [47]. In the NOD mouse conflicting results have been obtained. Hanafusa et al. [48] described aberrant expression of class II molecules prior to insulitis, as identified by anti-IA mouse antibodies, not only in the NOD but also to some extent in BALB/C and B10.GD mice. These results could not be confirmed using P7/7 rat MAb [23] which recognizes class-II molecules of b, d and k haplotype [49], and all islet cells appear negative with this antibody [25].

Immunotherapy

Cyclosporin A reduces insulitis in the NOD mouse but is unable to abolish it [50], while ICSA titres were similar or even higher than in control animals. In another report low-dose cyclosporin treatment has been shown to protect against insulitis [51]. These data indicate that cyclosporin A can partially suppress the cell mediated reaction but not the production of ICSA. Unfortunately the incidence of diabetes in cylcosporin treated animals was not investigated.

Nicotinamide, an inhibitor of poly-ADP-ribose synthetase, reduces the incidence of insulitis and diabetes in the NOD mouse [52]. Cyclophosphamide increases the incidence of diabetes but this effect can be blocked by nicotinamide [19]. ADCC is naturally elevated in the NOD mouse but falls after nicotinamide treatment [53]. This suggests an important role for ADCC and also indicates that nicotinamide has immunomodulatory properties. This is supported by the observation that single injections of nicotinamide prior to allogeneic islet transplantation prolong graft survival, a treatment which was much more successful if nicotinamide was combined with desferroxamine, an iron-chelating agent [54]. Nicotinamide seems to have some benefit in newly diagnosed Type 1 diabetic patients [55, 56] and increases C-peptide secretion in the first year after diagnosis [57].

Conclusion

The NOD mouse model shares a number of important characteristics with human Type 1 diabetes. The disease develops spontaneously and is not accompanied by

general immunodeficiency as in the BB rat. Differences include simultaneous lymphocytic infiltration of salivary glands and other organs, and a strong female predominance. Even so, study of mechanisms involved in insulitis, B-cell destruction, and the generation of other immunological disturbances allows hypotheses concerning human Type 1 diabetes to be developed and tested. The availability of high and low incidence lines may, in addition, offer clues to factors involved in the onset of diabetes.

Insulitis is in progress well before overt hyperglycaemia in the NOD mouse, and this is important for two reasons: (1) it allows the autoimmune process to be defined before complete B-cell destruction and hyperglycaemia have occurred. This might prove very useful in the search for new markers during this crucial phase of the natural history. (2) the prolonged and well defined prodromal period provides an excellent opportunity to test different approaches to immunotherapy early in the prediabetic stage.

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Appendix C

IMMUNOLOGY*today*

NOD mouse colonies around the world – recent facts and figures

Paolo Pozzilli, Alberto Signore, Alistair J.K. Williams and Philip E. Beales

Non-obese diabetic (NOD) mice are commonly used in autoimmune research. However, the diversity of these mice in developing autoimmune disease under different conditions prompted a group of researchers to compile a questionnaire on this subject. Here Paolo Pozzilli and colleagues comment on the results of this survey.

The world non-obese diabetic (NOD) registry was established in 1991 under the auspices of the Juvenile Diabetes Foundation (JDF) and the International Diabetes Immunotherapy Group with the aim of creating a data-base for comparing different NOD mouse colonies. NOD mice are susceptible to the spontaneous development of Type 1 diabetes, sharing many of the characteristics of the disease found in humans, and are currently the most widely used model for the study of the pathogenesis of Type 1 diabetes. A questionnaire was sent to those centres around the world known to work with this animal model.

The following comments aim to focus on any interesting and/or significant variations between the centres which co-operated in this venture. The data were collected during 1991–1992.

Colony size

It was clear from the data received that colony size varies quite markedly, some centres hold as few as ten breeders and others have more than 400. The same pattern can be observed in the number of ageing mice with figures ranging between 25 and 3 000.

Origin of breeding stock and breeding protocols

The origin of the breeding stock varies greatly. However, seven centres received stock from Dr E. Leiter (Jackson Laboratories, Bar Harbour, USA) while Merck, Sharpe and Dohme (Japan) and Clea (Japan) were each reported as suppliers to two centres. Mice mating times vary between centres, mice tend to be mated before the onset of IDDM at six to ten weeks. However, one centre cages its breeders together as early as three weeks of age, while others wait until the twelfth or even the twentieth week.

The different breeding protocols are reported in Table 1. The majority of centres use normoglycaemic breeders, but most centres consider diabetic pedigree when pairing. Out of the 22 centres questioned 20 used brother/sister mating with four centres maintaining distinct sublines. The average litter size is fairly uniform with a median of nine pups (range 1–16).

Eleven centres carry out genetic monitoring, the frequency of which varies greatly; only one centre

Table 1. Breeding protocols

	NB	DP	DM	ODP1	ODP2	ODM	ODF	RM
1	X			X	X			
2	X							
3	X					X	X	
4	X							X
5	X		X			X		
6	X				X			X
7		X	X					
8			X					
9								X
10	X							X
11		X						
12	X			X				
13	X							
14								X
15	X			X	X			
16				X	X			
17a	X							
b	X							
18			X			X		
19								X
20	X							X
21	X		X		X			
22				X	X			

NB: normoglycaemic breeders; DP: diabetic parents; DM: diabetic mother only; ODP1: offspring of one diabetic parent only; ODP2: offspring of two diabetic parents; ODM: offspring of diabetic mother only; ODF: offspring of diabetic mother; RM: random mating.
Notes: Centre 4 – new breeders are chosen from offspring of tail-grafted mice and usually selected from litters of >8 mice. Centre 20 – Production colony is selected for diabetic background while the foundation colony is maintained using brother–sister mating.

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Table 2. Cumulative incidence of diabetes (%)

Centre	At 20 weeks Males	At 20 weeks Females	At 30 weeks Males	At 30 weeks Females
1	1–5	20	10	65
2 Non-SPF	<5	<5	<5	20
SPF	<5	28	<5	60
3	15	30	30	70
4	22	46	41	78
5	8	88	10	90
6	8	40	25	85
7	5	15	24	80
8	<1		5	65
9			10–15	15–60
10		80		100
11	50	90	65	95
12	0	10	1	35
13 Wehi	<5	10	6	30–40
L+	20	60	20–30	80–90
14	5	20	35	60
15	3.9	29.2	15.4	55.4
16	6	40	15	18
17a	5.91	19.21	14.09	43.35
b	6.9	27.67	15.27	41.26
18	4	40	10	80
19	28	65	50	80
20	0	16	7	36
21	14	26	22	57
22	20.2	50	46	69

Table 3. Earliest recorded age of diabetes peak of incidence onset (weeks)

Centre	Onset Males	Onset Females	Peak Males	Peak Females
1	12	12	36	28
2	14 (SPF)	14 (SPF)	Non-SPF 35.5	Non-SPF 37
3	12	10	–	17–22
4	6	10	20–22	18–20
5	19	12	24–28	16–20
6	7	4	24	16
7	15	7	27	25
8	20	13	–	20–25
9	25	25	29	28
10	16	12	20–24	16–20
11	12	12	18	18
12	24	14	–	20–25.5
13	13	11.5	21.5	17
14	17	11.5	23–28.5	21.5–27
15	14	9	24	19
16	11	7	11	7
17a	15	9	16–24	16–20
b	12	11	26–20	12–20
18	16	13	27	21
19	14	12	27	27
20			28	23
21	17	11	16	16
22	11	8	16	16

monitors continuously, another monitors on a two to three yearly basis but the remainder tend to monitor every two to four months. Genetic monitoring is evaluated using several different parameters. Eight centres examine morphological characteristics; seven biochemical markers; eleven immunological markers, and six genetic markers. Other methods of evaluation include the examination of islets, and skin and tail grafting.

Diabetes diagnosis and incidence

Most centres screen for diabetes by urinary-glucose testing, and many centres also determine blood-glucose values. One centre tests for diabetes when wet cages are observed and another centre starts urine testing upon observing increased water consumption. Tests for urinary glucose generally commence between 6 and 14 weeks and are then repeated at weekly intervals. Blood-glucose testing is usually initiated at 6 to 12 weeks and repeated at intervals of between 1 and 4 weeks.

Cumulative incidence of diabetes

As expected, the cumulative incidence of diabetes at both 20 and 30 weeks is much higher for females than for males in all centres, except one where the cumulative incidence of diabetes at 20 weeks is very low (<5%) for both males and females (see Table 2). The highest cumulative incidence figures at 20 weeks were 50% for males and 90% for females. Two centres reported no diabetic male mice at all at 20 weeks. The incidence of diabetes in females ranges between 5% and 90% at 20 weeks. At 30 weeks the range is 20% to 100%, which is generally more than double the incidence at 20 weeks.

Age of onset and peak of incidence

Diabetes onset (see Table 3) occurs earlier in females (median 11.5 weeks, range 4–25 weeks) than in males (median 14.0 weeks, range 6–25 weeks). Interestingly, in four of the centres the earliest age of onset was the same for both male and female mice. The peak of diabetes incidence also occurs earlier in females (median 19.0 weeks, range 7–37 weeks) than in

males (median 24 weeks, range 11–36 weeks).

Environmental conditions/housing and maintenance of breeding stock

Questions concerning the housing and other factors which might affect the growth and development of the mice were also asked. Between 1 and 20 animals are kept per cage. Twenty one out of the 22 centres kept a constant room temperature (median 21.7°C, range 19–26°C). Most centres use an artificial light regime (11–14 hours per day), two centres employed natural lighting for 12 hours per day and one centre only allows its mice two hours of artificial light per day. Only four of the eight centres which look for seasonal variation in diabetes incidence observed a seasonal influence.

The protein, carbohydrate and energy values of the various diets used differed between centres. The protein content ranged from 14.7% to 27.5%; carbohydrate content from 4.5% to 71.5% and energy content from 290 Kcal/100g to 717 Kcal/100g. In general, all centres use the same feed for both stock and breeders, however two centres used a higher energy feed for breeders (see Table 4).

Table 4. Diets

Centre	Commercial name	% Protein	% CHO	Energy content /100g	Others
1	Nafag 900 (R&M)	26	5.5	345	
2	SD1	14.7	50	352	
3	UAR	17	?	290	
4	Old Guildford 96W	23	50–60	400	
5	Purina breeder	?	?	?	
6	Purina 500I	23.4	5.8	425	
7,22	Purina mouse	17.5	71.5	431	
8	CE2	24.8	51.6	345.2	
9	Tekland Premier	17	10	452	
10	?	24	4.5		
11	Agway 3500	22	?		4% fat
12	Dunstan Stock	18.2	58	304.5	5% fat
13	Barastoc	21.2	?	?	
14	Altromin 1434	23.5	53	?	
15	Quest 683 FFC(M)	19	43	318	
16	Oriental Yeast NMF	27.5	7.9	353	
17a	Sniff MR	21	64	347.5	
b	Sniff MR				
18	CLEA CL-2	24.3	48.5	340	
19	Zeigler (NIH-31)	18	?	?	
20	Lactamine R3	22	51.5	717	
21	Muricon-G-ster	21	68	450	

Pathogenic control and pathologies observed in NOD colonies

Seventeen of the 21 centres are pathogen controlled. Eleven observed infections in their colonies; there were four reports of bacteria, five of viruses and seven of parasites. Methods and frequency of testing for infection differed widely between centres. The most common approaches to the control of pathogens included the isolation/maximum barrier facility method and the sterilization of food and water.

Apart from insulitis, centres reported a number of other inflammatory lesions including sialitis and thyroiditis. Various tumours were observed in ageing mice including lymphomas and osteosarcomas (see Table 5).

Comments

This report shows that many differences exist between the different NOD mouse colonies throughout the world. Variations in diabetes incidence are perhaps the most important, since the NOD mouse is currently the most widely used model of Type 1 diabetes.

Some of the differences in diabetes incidence may be due to the different criteria used by the centres to define diabetes. In human Type 1 diabetes there is a strict definition of the disease contained in the WHO guidelines but no such standard exists for NOD mice. This lack of standardization however, can only explain some of the differences between colonies, suggesting that there are important genetic and/or environmental factors at work.

Breeding protocols tend to be similar for all centres, but housing

Table 5.

Tumours	Inflammatory lesions
Hepatomas	Adenitis
Lymphomas	Adrenalitis
Mammary carcinoma	Colitis
Mammary sarcoma	Haemolytic anaemia
Myoepitheliocarcinoma	Harderian/Lacrimal adenitis
Osteosarcomas	Intestinal nephritis
Osteochondrosarcoma	Insulitis
Rhabdomyosarcoma	Myositis
Thymoma	Meningitis
	Neuritis
	Osteomyelitis
	Parathyroiditis
	Sialitis
	Thyroiditis

conditions and other environmental factors vary greatly. These environmental differences may modulate the underlying immunological mechanisms leading to β-cell destruction or they may influence metabolic parameters leading to the expression of diabetes.

Variations in diabetes incidence may also stem from genetic differences between colonies. Significant genetic differences should not be expected since this is an inbred strain. However, two centres (13 and 17) keep mice from different sources, which although kept in similar environments, show differences in diabetes incidence, suggesting that some genetic drift has taken place.

Direct comparison of treatments between different centres is not always feasible because of the variations between colonies. A treatment which prevents diabetes in one centre may not always be successful when employed in another. At first sight this may appear to be a drawback, but the need to explain these differences may ultimately stimulate new theories and approaches for diabetes research in general and immunological studies in particular.

All new therapies aimed at preventing Type 1 diabetes should first be tested on animal models of the disease and the NOD mouse is one of the most appropriate models for this purpose. Variations in diabetes incidence do not disqualify the NOD mouse from being used in this role, since large variations also exist in the incidence of Type 1 diabetes in different human populations.

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IMMUNOPHARMACOLOGY

The June issue of *Immunology Today* will be a joint edition with *Trends in Pharmacological Sciences* focusing on the advances in immunopharmacology:

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